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Large Versus Small Unilamellar Vesicles Mediate Reverse Cholesterol Transport In Vivo Into Two Distinct Hepatic Metabolic Pools

Implications for the Treatment of Atherosclerosis

Wendi V. Rodriguez, Kiratin D. Mazany, Arnold D. Essenburg, Michael E. Pape, Thomas J. Rea, Charles L. Bisgaier, Kevin Jon Williams

Abstract Phospholipid liposomes are synthetic mediators of "reverse" cholesterol transport from peripheral tissue to liver in vivo and can shrink atherosclerotic lesions in animals. Hepatic disposal of this cholesterol, however, has not been examined. We compared hepatic effects of large (~120-nm) and small (~35-nm) unilamellar vesicles (LUVs and SUVs), both of which mediate reverse cholesterol transport in vivo but were previously shown to be targeted to different cell types within the liver. On days 1, 3, and 5, rabbits were intravenously injected with 300 mg phosphatidylcholine (LUVs or SUVs) per kilogram body weight or with the equivalent volume of saline. After each injection, LUV- and SUV-injected animals showed large increases in plasma concentrations of unesterified cholesterol, indicating mobilization of tissue stores. After hepatic uptake of this cholesterol, however, SUV-treated animals developed persistently elevated plasma LDL concentrations, which by day 6 had increased to more than four times the values in saline-treated controls. In contrast, LUV-treated animals showed normal LDL levels. By RNase protection

assay, SUVs suppressed hepatic LDL receptor mRNA at day 6 (to $61 \pm 4\%$ of control, mean \pm SEM), whereas LUVs caused a statistically insignificant stimulation. Hepatic HMG-CoA reductase message was also significantly suppressed with SUV, but not LUV treatment, and hepatic 7 α -hydroxylase message showed a similar trend. These data on hepatic mRNA levels indicate that SUVs, but not LUVs, substantially perturbed liver cholesterol homeostasis. We conclude that LUVs and SUVs mobilize peripheral tissue cholesterol and deliver it to the liver, but to distinct metabolic pools that exert different regulatory effects. The effects of one of these artificial particles, SUVs, suggest that reverse cholesterol transport may not always be benign. In contrast, LUVs may be a suitable therapeutic agent, because they mobilize peripheral cholesterol to the liver without suppressing hepatic LDL receptor mRNA and without provoking a subsequent rise in plasma LDL levels. (*Arterioscler Thromb Vasc Biol.* 1997;17:2132-2139.)

Key Words • atherosclerosis • HDL • gene expression • cholesterol • therapy

Four decades ago, the intravenous administration of aqueous dispersions of PL was shown to cause rapid, substantial shrinkage of lipid-rich arterial lesions in animals.¹ This striking finding has since been confirmed in a variety of experimental models of atherosclerosis (reviewed in Reference 2). Subsequent mechanistic studies indicated that dispersed PLs self-assemble into concentric spherical bilayers known as liposomes or vesicles.³ Furthermore, when intravenously injected at sufficient doses, initially cholesterol-free PL vesicles

remain intact in the bloodstream and are capable of extracting cholesterol from both lipoproteins and peripheral tissues.^{2,4} Thus, these circulating particles act as a sink for cholesterol, which is shuttled to them from tissues by HDL and other small acceptors of cholesterol.^{2,4-7} Because the liver serves as the predominant organ for the clearance of PL vesicles, it has been suggested that the antiatherogenic effects of these particles result from their ability to act as synthetic mediators of RCT from peripheral tissues to the liver.^{2,5}

Hepatic disposal of cholesterol transported from the periphery to the liver, however, is not well understood. Radioisotopic studies have suggested that cholesterol of HDL, the apparent natural mediator of RCT,⁸ is efficiently converted into bile acids by the liver and then excreted.⁹⁻¹² In contrast, direct measurements of sterol mass showed that accelerated delivery of cholesterol to the liver in vivo by apoE-rich HDL produces no change in biliary output of cholesterol or bile acids, but instead stimulates hepatic acyl-CoA:cholesterol acyltransferase and enhances VLDL secretion.¹³ Similar results, including LDL receptor suppression, have been reported in other situations involving cholesterol enrichment of hepatic cells.¹⁴⁻¹⁷ Moreover, intravenous infusion of apoA-I/PL disks into humans to enhance RCT causes a sustained rise in plasma levels of LDL.¹⁸ Taken together, these effects are more consistent with events leading to

Received February 29, 1996; revision accepted December 18, 1996.

From the Department of Biochemistry, Medical College of Pennsylvania, Philadelphia, Pa (W.V.R., K.J.W.); and Vascular and Cardiac Diseases, Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Co, Ann Arbor, Mich (K.D.M., A.D.E., M.E.P., T.J.R., C.L.B.). Dr Williams is now with the Division of Endocrinology, Jefferson Medical College of Thomas Jefferson University, Philadelphia, Pa.

Presented in part at the 68th Scientific Sessions of the American Heart Association, Anaheim, Calif, November 13-16, 1995, and published in abstract form (*Circulation*. 1995;92[suppl I]:I-556).

Correspondence to Kevin Jon Williams, Division of Endocrinology, Thomas Jefferson University, 1020 Locust St, Suite 349, Philadelphia, PA 19107-6799. E-mail: kevin.jon.williams@mail.tju.edu or Charles L. Bisgaier, Vascular and Cardiac Diseases, Parke-Davis Pharmaceutical Research, 2800 Plymouth Rd, Ann Arbor, MI 48105. E-mail: bisgaier@aa.wi.com.

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Selected Abbreviations and Acronyms

apo	= apolipoprotein
AU	= arbitrary units
CE	= cholesteryl ester
CEMP	= CE transfer protein
EC	= esterified cholesterol
LUV	= large unilamellar vesicle
MLV	= multilamellar vesicle
PL	= phospholipid
RCT	= reverse cholesterol transport
SUV	= small unilamellar vesicle
TC	= total cholesterol
UC	= unesterified cholesterol

the promotion, not the inhibition, of atherogenesis. Thus, depending on hepatic responses, not all agents that enhance RCT will be unambiguously beneficial.

In this study, we sought to determine the effect of liposomal structure on hepatic responses to enhanced RCT mediated by these synthetic particles. We compared LUVs (~120-nm), which have been shown to be catabolized in the liver primarily by Kupffer cells,¹⁹ versus SUVs (~35-nm), which are directed mainly to parenchymal cells.²⁰ Prior work has shown that both LUVs and SUVs mobilize cholesterol from lipoproteins and peripheral tissues, thereby enhancing RCT in vivo.⁶ The response of the liver to repeated injections of these two liposomal preparations over several days was determined by monitoring the concentrations of major plasma lipoprotein species and by measuring mRNA levels of key hepatic enzymes and proteins responsible for maintaining hepatic cholesterol homeostasis.

Methods

Materials

Egg phosphatidylcholine (>95% pure, Coatsome NC-10S) was purchased from Princeton Lipids. Human HDL₂ was isolated from fresh plasma by sequential ultracentrifugation (1.12 < d < 1.21 g/mL).²¹ All other chemicals and solvents were of analytical grade and purchased from Fisher Scientific Co.

Preparation of Vesicles

Three days before the start of the experiment in vivo, LUVs and SUVs were prepared at a concentration of 100 mg/mL. Six 6-g portions of solid egg phosphatidylcholine were each placed into 50-mL conical polypropylene centrifuge tubes, hydrated with 30 mL of filter-sterilized 150 mmol/L NaCl, 20 mmol/L HEPES, pH 7.4 (HEPES-buffered saline), and vortexed to generate MLVs. These preparations were kept overnight at 4°C. To generate LUVs, the MLVs were extruded 10 times under medium pressures (250 to 300 psi) through two stacked polycarbonate filters (100-nm pore size) that had been fitted into a 10-mL water-jacketed thermobarrel Extruder (Lipex Biomembranes). To generate SUVs, 30-mL batches of MLVs were each subjected to three 20-minute cycles of sonication in 50-mL round-bottom Pyrex glass tubes at 0°C under nitrogen (power setting=3, duty cycle=50%, Branson stud tip sonifier, VWR Co). The initially milky suspension clarified during this procedure. After sonication, batches were then centrifuged at 20 000g for 30 minutes to remove titanium shed from the probe during sonication.

The two vesicle preparations, LUVs and SUVs, were sterilized by passage through 0.45-μm Nalgene bottle-top filters, assayed for PL concentrations,²² and diluted with sterile HEPES-buffered saline to 100 mg PL per milliliter before injection. Consistent with prior literature, the diameters of the LUVs and SUVs generated were found to be 123±35 and

34±30 nm (mean±SD), respectively, determined by quasi-elastic light scattering using a Nicomp model 370 submicron laser particle sizer, equipped with a 5-mW He-Ne laser (Pacific Scientific).⁶

Experimental Design In Vivo

Normal 3- to 4-kg female New Zealand White rabbits (Hazelton Farms, Denver, Pa) were randomly distributed into three groups (n=4, LUV or saline treatment; n=3, SUV treatment). Approximately 3 mL of blood was collected from each animal via a medial auricular artery every morning during the study. Blood samples were immediately mixed with EDTA for anticoagulation (final concentration, 2 mmol/L in blood) and N-ethylmaleimide to inhibit lecithin:cholesterol acyltransferase (final concentration, 3 mmol/L).

On days 1, 3, and 5, right after the morning's blood collection, LUVs or SUVs (300 mg of PL per kilogram body weight), or the equivalent volume of HEPES-buffered saline, were bolus-injected into a marginal ear vein of each animal (approximately 10 mL per injection, infused over ~30 seconds). Immediately after blood collection on day 6, all animals were killed (100 mg pentobarbital per kilogram IV),²³ and several 100- to 200-mg liver samples were collected from each animal and snap-frozen in liquid nitrogen. Tissue samples were stored at -70°C until isolation of total RNA and lipid analysis.

Analyses of Plasma and Plasma Fractions

Triglyceride concentrations in whole plasma were determined by using a commercially available kit (Triglyceride G, Wako Chemicals USA, Inc). Whole-plasma concentrations of TC (equal to unesterified and esterified forms) and UC concentrations in whole plasma were directly determined enzymatically,²⁴ and EC was calculated by difference. Agarose gel electrophoresis of whole plasma followed by lipid staining with Sudan black²⁵ and rocket immunoelectrophoresis to quantitate whole-plasma-rabbit apoB, reported in Aβ^{27,28} were performed as previously described. Preliminary studies indicated that these rocket assays, which are performed in detergent,²⁶ are not affected by the presence of liposomes. Size distributions of plasma particles carrying TC and UC were determined by Superose 6HR high-performance gel chromatography (Rainin Instrument Co, Inc), including an on-line post-column analyzer, as previously described.^{27,28} Areas under the peaks were used to calculate the percent distribution of TC and UC corresponding to VLDL, LDL, and HDL size ranges in the elution profiles (Dynamax and Compare Module Software, Rainin Instrument Co, Inc, developed for Macintosh computers). Next, the absolute concentrations of TC and UC in each lipoprotein size range were determined by multiplying these percent distribution values by the independently determined TC and UC values in whole plasma. Absolute EC values in each lipoprotein fraction were determined by the difference between the calculated TC and UC in each lipoprotein fraction. Plots of the distributions of absolute lipid content by particle size were prepared similarly, i.e., the total area under each TC and UC elution curve from the on-line post-column analyzer was normalized to the corresponding whole-plasma assay result. Absolute EC distribution curves were then determined by difference.

Analytical Methods for Determination of Hepatic Lipids

Hepatic phosphatidylcholine, phosphatidylethanolamine, CE, UC, and triglyceride were extracted in the presence of 4-hydroxycholesterol internal standard, separated by silica column high-performance liquid chromatography, and then quantitated by an evaporative light-scattering detector, as previously described.²⁴ Notice that these tissue CE determinations include the mass of both the steryl and fatty acyl moieties, whereas the EC measurements in plasma and plasma fractions include only steryl mass.

Hepatic mRNA Analysis

Rabbit cDNAs encoding the LDL receptor, HMG-CoA reductase, 7 α -hydroxylase, and CETP were prepared in polymerase chain reactions using primers based on phylogenetically conserved sequences and then cloned into the pBluescript II SK (+) plasmid (Stratagene Cloning Systems). The identity of each cloned cDNA was confirmed by sequencing, as described in detail by Rea et al.²⁹ For each cloned cDNA, an unlabeled sense mRNA product, to be used as an internal standard, and a labeled antisense mRNA probe were synthesized. Both synthetic mRNAs were designed to contain the partial coding sequence from rabbit, linked to a short sequence from the plasmid.

Total RNA was isolated from the snap-frozen rabbit liver samples using RNazol (Cienna/Biotec Inc), quantified by A_{260} , and assessed for degradation by agarose electrophoresis. Messenger RNAs of interest were quantified by an internal standard/RNase protection assay, as described previously in detail.³⁰ Each protection assay contained (1) 30 μ g of total liver RNA, (2) the synthetic unlabeled sense mRNA internal standard (30 pg LDL receptor, 10 pg HMG-CoA reductase, 10 pg 7 α -hydroxylase, or 5 pg CETP), and (3) 1.0 ng of the synthetic radiolabeled antisense mRNA probe (specific activity, 2×10^6 cpm/ μ g). After digestion with RNase, protected mRNA probes were quantified by polyacrylamide gel electrophoresis and then autoradiography using a PhosphorImager (Molecular Dynamics). Probes protected by authentic versus internal-standard mRNA differed in molecular weight by the size of the incorporated plasmid sequence and were directly identified by control reactions in which either rabbit liver RNA or the unlabeled internal standard mRNA was individually omitted. The ratio of radioactivity in the two protected bands, multiplied by the known amount of internal-standard mRNA and corrected for molecular weight differences between authentic and internal-standard mRNA, gave the mass of hepatic liver mRNA of interest.

Protein Uptake by Large and Small PL Vesicles In Vitro

To determine whether there are systematic differences in the acquisition of proteins by LUVs versus SUVs, these particles were incubated in vitro for 4 hours at 37°C with HDL, using a PL ratio of 5:1 (vesicle:HDL), which is similar to ratios achieved in vivo and does not disrupt vesicle structure.³¹ PL vesicles were then separated from the HDL by passage over a 130 \times 15-cm column of Sepharose CL-6B. Purity of these modified liposomes was verified by the absence of detectable esterified cholesterol³² by gas-liquid chromatography.^{33,34} The modified LUVs and SUVs were analyzed for protein content by modified Lowry,³⁴ PL content enzymatically,³⁵ and protein species by SDS-polyacrylamide gel electrophoresis.

Statistical Analyses

ANOVA was used to compare the three experimental groups. When ANOVA indicated differences among the groups, pairwise comparisons between groups were performed using the Student-Neumann-Keuls q statistic.³⁶ Unless otherwise indicated, all results are displayed as mean \pm SEM, ($n=4$, LUV and saline groups; $n=3$, SUV group). Absent error bars in figures indicate SE values smaller than the drawn symbols.

Results

Alterations in Plasma Lipids and Lipoproteins in Response to Repeated Injections of PL Vesicles

Three intravenous injections of PL into normal rabbits caused large increases in the plasma concentrations of UC, indicating mobilization of tissue stores (Fig 1A), similar to previous findings.^{1,2,4,5,27} LUVs had a larger effect than SUVs on plasma UC concentrations, consis-

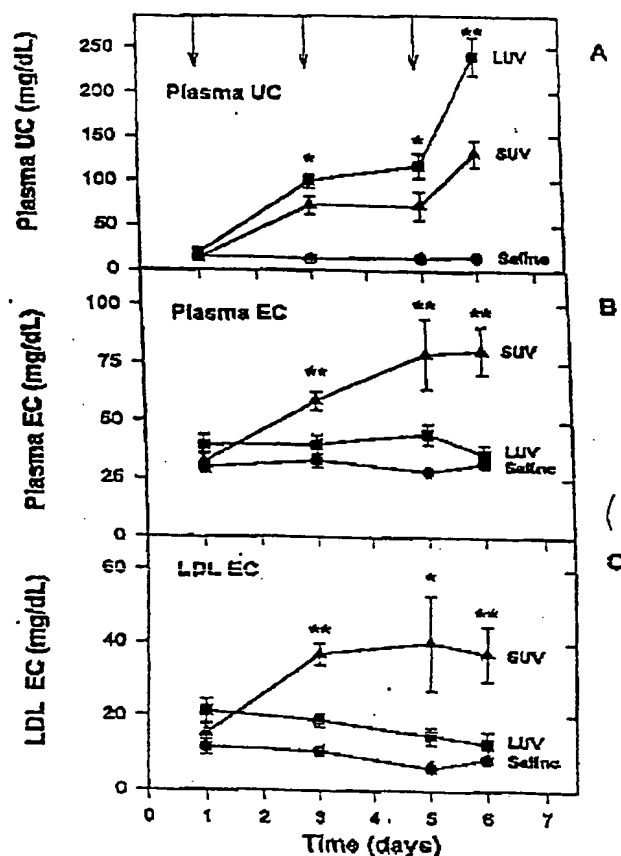


Fig 1. Plasma unesterified cholesterol (A), plasma esterified cholesterol (B), and LDL esterified cholesterol concentrations (C) in New Zealand White rabbits after repeated injections of LUVs (■) or SUVs (▲) at a dose of 300 mg/kg, or the equivalent volume of saline (●). Arrows indicate intravenous injections of PL vesicles or saline on days 1, 3, and 5. Plasma samples on those days were obtained immediately before the injections. Each data point displays the mean \pm SEM. In B and C, LUV values were statistically indistinguishable from the corresponding control (saline) values, except for C, day 3 ($0.1 < P < 0.05$). In all three panels, SUV values after day 1 were greater than control ($P < 0.01$). * $P < 0.05$, ** $P < 0.01$; significant differences between LUV and SUV values.

tent with the prior observation that per milligram of administered PL, LUVs mobilize cholesterol more efficiently.⁶ SUVs caused a rise in plasma concentrations of EC, confirming prior reports of this effect after hepatic uptake of cholesterol mobilized by these particles.^{4,5} Surprisingly, although LUVs mobilize more cholesterol to the liver, there was no effect at all on plasma EC concentrations (Fig 1B).

Because EC cannot be transported to any great extent by PL vesicles owing to their lack of a hydrophobic core, we sought to determine which lipoprotein carried the extra plasma EC after SUV injection. Gel filtration revealed that most of the increase in whole-plasma EC was transported by LDL-sized particles (Fig 1C, Table 1, and Fig 2A). Under these conditions, liposomal remnants would not appear in this size range, because

TABLE 1. Unesterified and Esterified Cholesterol Concentrations in Whole Plasma and in VLDL-, LDL-, and HDL-Sized Particles on Day 6

Treatment	Whole Plasma		VLDL		LDL		HDL	
	UC	EC	UC	EC	UC	EC	UC	EC
LUV	239.8±20.5*	36.1±4.1	207.0±20.7*	2.0±1.9	21.8±0.7*	12.6±3.3	11.0±0.5	21.8±1.4
SUV	131.4±14.7*	80.7±10.3*	51.4±4.4†	6.3±1.9	61.1±9.1*	37.1±7.5*	19.0±1.2*	37.3±2.1*
Saline	15.3±0.8	31.8±2.0	0.9±0.2	0.5±0.1	4.2±0.4	8.4±0.7	10.2±0.6	23.0±1.7

All values are mean±SEM expressed as milligrams lipid per deciliter plasma. Each LUV value was significantly different from the corresponding SUV value ($P<0.01$), except for VLDL EC (not significant).

* $P<0.01$; † $P<0.05$; significantly different from saline control.

vesicle structure is stable in plasma at 300 mg/kg^{4,21} and remnants that form at lower vesicle doses are exclusively PL-apoprotein disks that coelute with HDL.^{31,32} Quantitatively, SUVs increased plasma concentrations of LDL EC to over fourfold compared with saline control (Table 1), whereas injections of LUVs caused a small but statistically insignificant decrease during the study (Fig 1). Rabbit apoB assays of plasma from day 6 showed a trend toward higher values in the SUV group ($0.31±0.04$ AU) than the LUV group ($0.16±0.07$ AU). These results suggest a proportionately greater increase in LDL EC than in plasma apoB after SUV administration, raising the possibility of increased LDL size, which was confirmed by a slight shift on the EC elution profile (Fig 2A, EC profile after SUV treatment). No shifts were evident in any HDL EC peaks (Fig 2A). The SUV-mediated increase in LDL concentration was confirmed by agarose gel electrophoresis followed by Sudan black staining,²⁵ which revealed a darker but otherwise unremarkable β -band (Fig 2B). As previously reported,⁴ the SUVs in plasma exhibited a mobility ahead of LDL owing to their acquisition of plasma proteins, chiefly from HDL. In contrast, plasma LUVs exhibited essentially the same mobility as freshly prepared, protein-free vesicles, ie, just above the origin (Fig 2B; discussion to follow).

Fractionation data for all particle sizes from the final bleed on day 6 are summarized in Table 1. Notice that the additional UC mobilized into the plasma of LUV-treated animals was mainly confined to the VLDL size range, while the additional UC in the SUV-treated animals was in both the VLDL and LDL size ranges, consistent with the smaller size but greater heterogeneity of SUVs compared with LUVs (see "Methods"). Also, lipoprotein fractionation revealed an increase in the concentration of HDL EC in SUV-treated animals. This increase in HDL EC, however, represented only a small fraction of the total cholesterol mass mobilized by the injected vesicles. No significant changes in plasma concentrations of triglycerides were observed (data not shown), consistent with prior studies.³⁷

Alterations in Hepatic Lipids and mRNA in Response to Repeated Injections of PL Vesicles

On day 6, 24 hours after the third injection, hepatic samples were taken for lipid and mRNA analyses. Because vesicles in the bloodstream eventually achieve a molar ratio of UC:PL of about 0.8,⁴ which is far higher than the ratio of 0.12 found in normal hepatic tissue (Table 2), we anticipated that hepatic uptake of vesicles might increase this ratio in liver. Both types of PL vesicles did substantially increase the hepatic UC:PL ratio (Table 2; $P<0.01$), consistent with liposomal deliv-

ery of cholesterol mass to the liver. With both types of particles, the significant increase in the hepatic UC:PL ratio appeared to result from increased hepatic UC and decreased hepatic PL, although only the SUV-induced decrease in hepatic PL reached statistical significance compared with saline control. No effects on hepatic triglyceride content were observed (data not shown).

Hepatic mRNA levels for key enzymes in cholesterol homeostasis are shown in Fig 3. The three injections of SUVs caused ~40% to 50% suppression of hepatic mRNA levels for the LDL receptor and HMG-CoA reductase. A similar though statistically insignificant trend was seen with 7 α -hydroxylase. These results are consistent with regulatory effects seen after substantial cholesterol loading of parenchymal cells, which compensate by suppressing their uptake of LDL and their synthesis of sterol (see References 15, 17, 39, and 40). In contrast, the three injections of LUVs caused increases, though statistically insignificant, in each of these messages. Finally, LUVs, but not SUVs, significantly suppressed hepatic mRNA for CETP.

Protein Uptake by Large and Small PL Vesicles In Vitro

Based on the electrophoretic mobilities in Fig 2B, we sought to quantitate the acquisition of proteins by LUVs versus SUVs. After incubation with HDL, LUVs acquired 1.09 μ g of protein per milligram of liposomal PL, whereas SUVs acquired 40.4 μ g/mg, ie, almost 40 times as much. Consistent with prior literature,^{4,41} SDS-polyacrylamide gel electrophoresis confirmed that the major protein acquired by both LUVs and SUVs was apoA-I (data not shown).

Discussion

In the current study, we have demonstrated that the metabolic effects from several days of enhanced RCT in vivo strongly depend on the particle that carries cholesterol from the periphery to the liver. Furthermore, RCT mediated by some particles, such as SUVs (see "Results") and HDL-like complexes (see References 13 and 18), can produce metabolic consequences that may not be benign.

Our results with SUVs are entirely consistent with prior literature concerning cholesterol enrichment of hepatocytes. When liver cells were cholesterol enriched in vivo by a single injection of apoE-rich HDL that was rapidly cleared from plasma into liver⁴² or by feeding nonhuman primates long term on atherogenic diets,³⁹ similar results were observed, including LDL receptor suppression and lipoprotein oversecretion. Similarly, cholesterol enrichment of hepatocytes in vitro causes suppression of LDL receptors^{42,43} and HMG-CoA re-

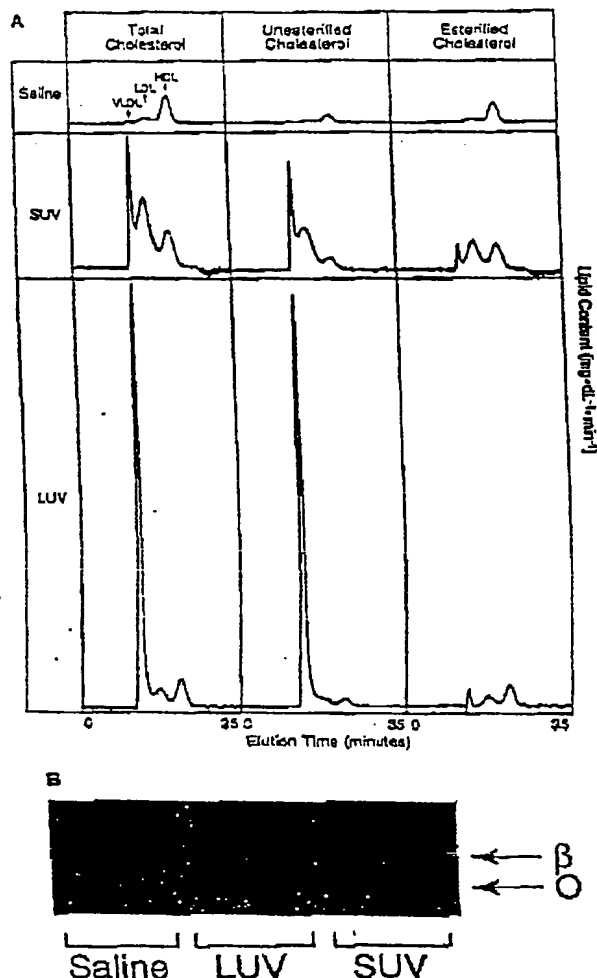


Fig 2. Fractionations of plasma from day 8 after repeated injections of LUVs, SUVs, or saline. A, Distributions of lipids in whole plasma by particle size. Plasma samples were gel filtered through a Superose 6HR column, and eluents were assayed for TC and UC. The total area under each curve was normalized to the whole-plasma assay for TC or UC. The EC curve was generated by difference. Plots from one animal in each group are shown, with all panels drawn to the same scale. Specific lipoproteins are identified in the upper left-hand plot. The area under the LDL EC peaks in the saline, SUV, and LUV animal were the same, much larger, and slightly smaller, respectively, than on day 1. B, Distributions of plasma lipids by particle charge. Four-microliter plasma samples from two animals in each group at day 8 were electrophoresed through 0.5% agarose and then stained with Sudan black. O indicates origin and β , migration of an LDL standard.

ductase,^{44,47} as well as enhancement of apoB secretion.^{45,48,49} The expression of hepatic 7α -hydroxylase is stimulated by cellular cholesterol enrichment in rats⁵⁰ but inhibited in rabbits,^{17,40} consistent with the trend in Fig 3. Thus, RCT by these synthetic SUVs was associated with events that are entirely consistent with known molecular consequences of cellular cholesterol enrichment: sterol-responsive messages in the liver, such as the

TABLE 2. Lipid Content in Livers of Animals on Day 8 of Treatment With LUVs, SUVs, or Saline

Treatment	UC	CE	PL	UC:PL Ratio (mol/mol)
LUV	5.3 \pm 0.2	1.6 \pm 0.6	65.0 \pm 1.6*	0.16 \pm 0.01†
SUV	4.6 \pm 0.5	2.8 \pm 1.1	53.1 \pm 3.0†	0.16 \pm 0.02†
Saline	4.3 \pm 0.2	2.9 \pm 1.2	68.3 \pm 1.5	0.12 \pm 0.01

Mass values are shown as mean \pm SEM, expressed as microgram lipid per milligram protein in liver (n=4 animals per treatment group). Phospholipid content equals phosphatidylcholine plus phosphatidylethanolamine.

* $P<.01$; significant differences between LUV and SUV values.

† $P<.01$; significantly different from saline control.

LDL receptor, were suppressed, and consequently, plasma LDL concentrations rose. It is nonetheless surprising that the metabolic effects on the liver of RCT mediated by SUVs in the current study or by apoE-rich

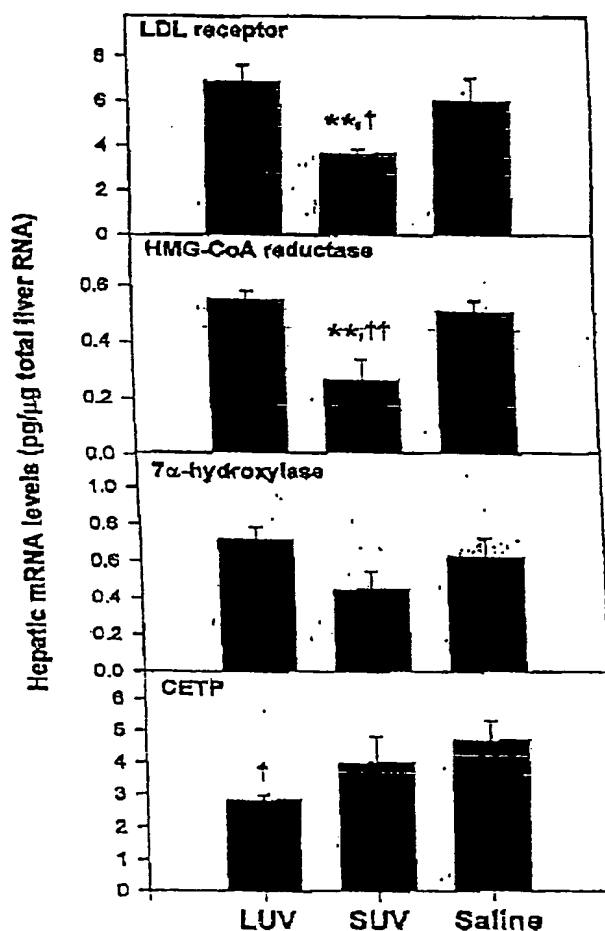


Fig 3. Hepatic mRNA levels for the LDL receptor, HMG-CoA reductase, 7α -hydroxylase, and CETP, expressed as picograms of specific mRNA per microgram total liver mRNA. Messages in liver samples obtained at day 8 were quantified by RNase protection assay. Each data point displays the mean \pm SEM. * $P<.05$, ** $P<.01$; significant differences between LUV and SUV values. † $P<.05$, †† $P<.01$; significantly different from saline control.

particles in a previous study¹³ appear to be inconsistent with antiatherogenic actions.

Our results with LUVs, however, were starkly different. Prior work has shown that LUVs transport significantly more peripheral cholesterol *in vivo* to the liver than do SUVs,⁶ and yet there was no suppression of sterol-responsive messages in the liver and no rise in plasma LDL concentrations (Figs 1 through 3). We propose three possible explanations for the difference in metabolic response to LUVs versus SUVs. First, it has been reported that LUVs are taken up by Kupffer cells,¹⁹ whereas SUVs are primarily directed toward hepatic parenchymal cells.²⁰ Presumably, this is partly a mechanical consequence of hepatic architecture: hepatic endothelial fenestrae are oval openings of about 100×115 nm,^{21,22} through which SUVs of 35-nm diameter can readily pass and gain access to parenchymal cells. LUVs of 120-nm diameter or slightly larger will not pass easily and are cleared instead by the macrophage Kupffer cells that line liver sinusoids. While SUVs also have access to Kupffer cells, their sheer number (~10 times as many SUVs as LUVs per milligram of PL) appears to saturate the reticuloendothelial system, and so parenchymal cells predominate in their clearance (see Reference 20).

Cholesterol-clearance pathways mediated by parenchymal versus Kupffer cells are likely to have distinct metabolic consequences. Direct delivery of cholesterol to parenchymal cells by SUVs would be expected to suppress sterol-responsive messages. Delivery of cholesterol to Kupffer cells can be followed by gradual transfer of lipid to parenchymal cells,^{19,23} for example, via the extensions of Kupffer cells that reach down through the space of Disse to make physical contact with parenchymal cells.²³ The rate of sterol delivery to the parenchymal cells by transfer from Kupffer cells can be slower than by direct uptake; the chemical form of the sterol may be altered by the Kupffer cells before transfer (see Reference 54); and, on the basis of other pathways for lipid transfer among liver cells,²⁴ the process of transfer from Kupffer to parenchymal cells may be regulated, whereas SUV clearance does not appear to be.²⁵

The second possible explanation for the difference in metabolic response to LUVs versus SUVs is based solely on differences in the kinetics of their delivery of cholesterol to the liver. In mice, LUVs are cleared from plasma somewhat more slowly than are SUVs and thereby produce a relatively constant delivery of cholesterol mass to the liver from the time of injection until the bulk of injected material is cleared.⁶ Similarly, in rabbits, LUVs are cleared with a $t_{1/2}$ of ~27 hours (W.V. Rodriguez, M.J. Hope, unpublished studies, 1997), whereas SUVs are cleared more rapidly,⁵ thereby delivering a large bolus of cholesterol mass to the liver between 4 and 12 hours after injection, which is followed by a rise in plasma EC concentration.⁵ The slow, steady delivery by LUVs may avoid disrupting hepatic cholesterol homeostasis, while the more rapid uptake of SUV cholesterol may overwhelm the ability of the liver to maintain homeostasis, thereby provoking suppression of hepatic LDL receptors (see Reference 15).

The third possible explanation is based on the striking quantitative difference in protein adsorption between the two types of vesicles (Fig 2B and "Results"), which is presumably a result of their distinct surface curvatures.²⁶ Thus, it is conceivable that SUVs, but not LUVs, would

avidly strip apoE from VLDL, thereby slowing its clearance from plasma and favoring its conversion to LDL (see Reference 56). This scenario, however, would not explain the divergent effects of the two types of vesicles on hepatic gene expression (Fig 3). Alternatively, differences in adsorbed apoproteins might play a role in directing the PL vesicles into different hepatic metabolic pools, although there is no direct evidence that apoproteins mediate hepatic uptake of these particles.²⁷

Our results with hepatic CETP message were unexpected; namely, suppression by LUV injections and no significant effect of SUV injections, despite cholesterol delivery. Notice that CETP mRNA differs from the other three messages that we studied: it is equally distributed between hepatic parenchymal and nonparenchymal cells on a per-gram basis, whereas hepatic mRNAs for the LDL receptor, HMG-CoA reductase, and 7 α -hydroxylase are >90% in parenchymal cells.²⁸ Prior reports indicate that hyperlipidemia in rabbits is associated with increases in plasma CETP mass and hepatic message,²⁸ although separate effects on parenchymal and nonparenchymal cells are not known. Also, suppression of CETP is usually followed by increases in HDL EC,²⁹ which we did not see here after LUV injections. Our results may not be directly comparable to the study by Quinet et al,²⁸ however, because we investigated the redistribution of endogenous cholesterol among tissues by artificial particles, whereas the prior work in animals involved enhanced dietary intake. Thus, it is likely that LUVs suppressed hepatic CETP mRNA by depleting cholesterol from a regulatory pool that ordinarily stimulates CETP production and then disposed of this cholesterol into a nonstimulatory pool. SUVs, which are cleared differently, produced no overall effect on CETP message. Because the role of CETP in provoking or preventing atherosclerosis is controversial,⁴⁰⁻⁴² the importance in atherogenesis of our finding that CETP mRNA is suppressed after LUV injections is not clear. Nevertheless, because LUVs and SUVs produced different effects on CETP mRNA, as well as on the other messages, there is a consistent pattern of divergent regulatory effects between these two synthetic mediators of RCT.

For many reasons, the safe enhancement of RCT is an important medical goal. First, it has been recently accepted that most human heart attacks are caused by rupture-prone lesions that are rich in lipid and foam cell macrophages.^{43,44} One goal for treatment of preestablished disease is to stabilize these lesions. Apparently, these lesions can be gradually rendered less dangerous by aggressive lipid-lowering therapy,⁴⁵ which presumably reduces lesional content of lipid⁴⁶ and possibly tissue factor.⁴⁶ We speculate that massive enhancement of cholesterol transport from peripheral tissues to the liver by LUVs *in vivo* should achieve the same beneficial result quickly and directly. Second, enrichment of vessel wall cells with cholesterol or oxidized derivatives is known to produce substantial dysfunction.⁴⁶⁻⁴⁹ Cholesterol-enriched endothelial cells lose their ability to produce endothelial-derived relaxing factor,^{50,51} and cholesterol-enriched smooth muscle cells exhibit supranormal levels of cytosolic calcium⁵² and enhanced proliferation.⁵³ Many of these effects can be quickly reversed *in vitro* by removal of the excess cellular UC.⁵⁴ We speculate that enhanced RCT should accomplish the same *in*

vivo, with rapid therapeutic benefit. Third, platelet hyperactivity can be caused by an increased platelet membrane ratio of UC:PL, which has been described in some human hyperlipidemias.⁷⁵⁻⁷⁸ Again, a massive enhancement of RCT might produce a rapid therapeutic benefit in this situation by reducing platelet reactivity in vivo.

Overall, our findings indicate that these synthetic particles, LUVs and SUVs, mediate RCT in vivo, though with markedly different regulatory effects on the liver. LUVs appear to be the better therapeutic agent, because they mobilize cholesterol from the periphery to the liver without suppressing hepatic LDL receptor message and without provoking a rise in plasma LDL concentrations.

Acknowledgments

W.V. Rodriguez was an International Research Fellow of the American Heart Association and then supported by a Research Fellowship from the Heart and Stroke Foundation of BC and Yukon, Canada. K.J. Williams is an Established Investigator of the American Heart Association/Genentech. We wish to thank Dr Reynold Homan for assistance with hepatic lipid analyses and Dr Michael C. Phillips for very helpful discussion and suggestions.

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